CHEMICAL BASIS FOR PHOTOMUTAGENICITY IN SYNTHETIC FUELS

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ABSTRACT

Photomutagens (chemicals that enhance the mutagenicity of near UV-visible radiation) have been detected in a variety of experimental coal- and oil shale-derived synthetic fuels using S.typhimurium strain TA98 and fluorescent light. In this study, photomutagenic activity was measured among synfuel samples that included crude and hydrotreated shale oil, coal oil distillation fractions, and chemical class fractions of coal and shale oils. Photomutagenic activity was found to increase with increasing boiling point and was concentrated in fractions enriched in neutral polycyclic aromatic hydrocarbons (PAH). These results point to high molecular weight neutral PAH constituents as important photomutagenic components among the samples tested. The photomutagenic activities of the materials tested correlate well with the previously reported tumorigenic activities of the same samples on mouse skin but correlate poorly with the previously reported mutagenic activities in the conventional Salmonella/mammalian-microsome mutagenicity test in which neutral PAH fractions were inactive.

INTRODUCTION

A need currently exists for developing alternative energy technologies such as producing petroleum substitutes from coal and oil shale. However, the elevated risk for skin cancers seen among past coal and shale oil production and maintenance workers (1,2) points to a potential problem that must be addressed by the developing synthetic fuels technologies. The hazards associated with synfuel processes and materials should be identified and minimized prior to commercial production. Accurate toxicological data is, therefore, needed for risk assessment purposes. In addition, inexpensive and rapid tests that can predict tumor initiating, tumor promoting, co-carcinogenic and anticarcinogenic activities among synfuel samples are needed due to the potentially large number of feedstocks, conversion processes and process streams.

The S. typhimurium histidine reversion bioassay (the Ames assay) (3,4) utilizing strain TA98 and a rat liver enzyme preparation has been only partially successful as a short-term bioassay for detecting tumor initiators and/or complete carcinogens in synfuels as mutagens. Although positive correlations between the microbial mutagenicity and the relative tumor initiating capacity of synfuels and related materials have been observed (5,6), "false negatives" have been reported. For example, a hydrotreated Paraho shale oil has been reported to be carcinogenic (7-10) but not mutagenic

(9-12). Microbial mutagens and carcinogens have both been reported to be concentrated in the highest boiling distillation fractions from synthetic fuels (5, 12-19). However, the microbial mutagenicity of synfuel chemical class fractions has been found to reside primarily in fractions enriched in amino-substituted polycyclic aromatic compounds (20,21). Neutral polycyclic aromatic hydrocarbon-enriched synfuel fractions, which have shown relatively high carcinogenic activity, have demonstrated little or no microbial mutagenicity (17,22). Based upon these latter results, Holland et. al. (22) have suggested that with synfuels, the "apparent correlation between Ames mutagenicity and mammalian skin tumorgenicity may be due to the coincidental occurrence of two dissimilar classes of molecules."

Coal- and shale-derived liquids have been known to possess potent phototoxic properties for many years (i.e., they enhance the lethality of non-ionizing radiation) (23). In addition, a positive correlation has been reported to exist between the phototoxic and carcinogenic activities of polycyclic aromatic compounds (24-27). More recently, a number of synfuel materials have been shown to possess photomutagenic activity, i.e., the ability to enhance the mutagenicity of non-ionizing radiation (11,28-34). However, the significance of this photomutagenic activity towards potential occupational hazards and the role of photomutagens in effects seen in mammals are not yet clear. In an effort to determine the chemical nature of photomutagens in synthetic fuels and to determine the relationship between photomutagenic and carcinogenic activities, we have begun testing synfuel fractions (isolated based upon boiling point and chemical class) that have been chemically characterized and tested for skin tumor initiating capacity. We report here our progress to date and preliminary conclusions.

MATERIALS AND METHODS

Samples

Samples included coal-derived materials from the Solvent Refined Coal-I (SRC-I) and SRC-II processes. An SRC-II blend of recycle process solvent and atmospheric flash column bottoms was collected at the Harmarville, PA process development unit and was distilled by Gulf Science and Technology Co. into the following boiling point cuts (^{o}F): 300-700, 700-750, 750-800, 800-850, and 850+. An SRC-I process solvent was collected from the Wilsonville, AL pilot plant operated by Catalytic, Inc. and Southern Company Services and was distilled by Air Products, Inc. (Linwood, PA) into boiling point cuts which included an 800^{o}F+ cut, the only SRC-I cut used in this study. (Sample descriptions are summarized in ref. 17).

A crude Paraho shale oil and a hydrotreated Paraho shale oil were obtained from the Oak Ridge National Laboratory Synfuels Research Materials Repository (Repository ref. # 4601 and 4602, respectively). The crude shale oil was produced by the Paraho Development Corp. at Anvil Points, CO and hydrotreated at Sohio's Toledo (OH) refinery. The densities of these samples are reported to be 0.917 g/ml (crude) and 0.842 g/ml (hydrotreated) (44). A more complete description of the shale oil samples is available in ref. 35.

The coal oil and shale oil samples were acquired from pilot plant or process development unit test facilities and should not

necessarily be considered to be representative of synthetic fuel materials that may eventually be produced at commercial-scale facilities.

Chemical Class Separation

The hydrotreated Paraho shale oil sample was chemically fractionated prior to bioassay using the procedure of Later et al. (36). The separation procedure involved adsorption of approximately 0.1-0.2 g of sample onto 3 g of neutral alumina, packing the alumina onto 6 g of fresh alumina, and successive elution with hexane (fraction A1), benzene (A2), chloroform:ethanol (99:1)(A3) and 10% ethanol in tetrahydrofuran (A4) to give fractions enriched in aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAH), nitrogen-containing polycyclic aromatic compounds (NPAC) and hydroxy-substituted polycyclic aromatic compounds (HPAC), respectively. The coal-derived samples were fractionated using a slightly modified procedure which has been routinely used at Pacific Northwest Laboratory to generate fractions for chemical and biological analysis. The alumina was activated by storing at 150°C, the water content of the alumina was maintained at approximately 1.5% and methanol replaced ethanol:tetrahydrofuran as the final eluent. Also, the sample-adsorbed (3g) and fresh alumina (11g) were solvent-packed with hexane (37,38). It should be noted that oxygen and sulfur heterocyclic compounds elute primarily with the neutral PAH-enriched fraction (A2) using these procedures (36).

Preparation of Samples for Photomutagenicity Testing

Synfuel samples were diluted and mixed in DMSO (Sigma, Grade 1) prior to bioassay. Most of the materials tested, including the A2-A4 chemical class fractions, were completely soluble in DMSO; however, when some of the coal oil distillate cuts were diluted in DMSO, a small amount of insoluble material was observed. A considerable portion of the crude and hydrotreated shale oils appeared to be DMSO-insoluble. In all cases the DMSO-insoluble components had the same appearance (waxy, aggregating particles) as the aliphatic hydrocarbon fractions (A1) diluted in DMSO. The insoluble materials present in the DMSO preparations of the SRC crude distillate cut materials and the shale oils were assumed to be the aliphatic hydrocarbon components. The aliphatic hydrocarbon fraction weight percent compositions of the SRC-II 300-700°F, 700-750°F, 750-800°F, 800-850°F and 850°F+ distillates have been reported to be 32, 15, 15, 4 and 2%, respectively (39). The crude and hydrotreated Paraho shale oils have been reported to contain 45 and 16% DMSO-extractable material, respectively (29). An aqueous solution of an aliphatic hydrocarbon-enriched (A1) fraction was prepared using the detergent Tween 80 (Sigma) and methods previously described (40-42). In brief, an aqueous preparation was made with the A1 fraction at 2 mg/ml and 20% (v/v) Tween 80 in distilled water. Bacterial suspensions were exposed to the test substances by adding 0.1 ml of the aqueous or DMSO preparation to 3.9 ml of bacterial suspension, giving final treatment concentrations of either 2.5% DMSO (v/v) or 0.5% Tween 80 (v/v). In treatments with DMSO preparations having an insoluble aliphatic hydrocarbon component, the actual oil concentration in solution would be reduced in proportion to the amount of DMSO-insoluble (aliphatic) material present.

Bioassay

Photomutation assay procedures were performed essentially as described previously (11) and were slight modifications of the method of Ames et al. (3) and Maron and Ames (4). Suspensions of Salmonella containing 1-2x10^9 cells/ml in phosphate buffer (0.1 M, pH 7.4) were untreated or treated with 1) fluorescent radiation, 2) test substance (in the dark), or 3) fluorescent radiation and test substance, concurrently. Fluorescent radiation was from General Electric and Philips (Westinghouse) 15 Watt cool white fluorescent tubes with an irradiance to the suspensions of 18 W/m^2. (The irradiance was 17 W/m^2 in tests of the unfractionated shale oils.) Following predetermined durations of exposure to test substance and/or radiation, 0.1 ml volumes of treated suspensions were removed for measurements of mutation (as reversion to histidine prototrophy) or survival. Histidine reversion was measured using the plate incorporation method (3) with top agar supplemented with 0.1 ml of nutrient broth and insufficient histidine for growth of non-revertants to macroscopic colonies. Survival was measured following dilution in nutrient broth by plating 0.1 ml volumes using the plate incorporation method (3) with top agar supplemented with 0.15 ml of 0.1 M histidine HCl (Sigma). Strain TA98 was used in all experiments.

The Salmonella/mammalian-microsome test procedure of Ames et. al. (3) and Maron and Ames (4) was used with minor modifications as described in ref. 33. Strain TA98 was used in all experiments.

Revertant and surviving colonies were counted following 2-3 days incubation at 37.5°C. The spontaneous number of revertants/plate observed in the absence of treatment was subtracted from revertant/plate values observed on mutation assay plates to give corrected revertant/plate values for each treatment. Based upon the number of corrected revertants/plate and the corresponding number of survivors for each treatment, the mutation frequency response (in revertants/10^9 survivors) was calculated by the method of Green and Muriel (43). The mutation frequency response to fluorescent light (in the absence of test substance) was subtracted from the mutation frequency responses to light and test substances to give the corrected revertants/10^9 survivors (plotted in the text figures). Text Figures 2 and 3 and Tables 1 and 2 give means and standard deviations for values obtained from multiple, indpendent experiments (where they can be plotted).

RESULTS

Effect of Hydrotreatment on the Mutagenicity of Shale Oil

Crude and hydrotreated Paraho shale oil samples were tested for mutagenicity 1) in the Salmonella/mammalian-microsome test and 2) in the photomutation assay. When the parent crude and hydrotreated oils were tested in the Salmonella/mammalian-microsome test in the absence of microsomal enzymes and in the photomutation assay in the absence of light, mutagenic responses to the oils were not detected; direct-acting mutagens were not detected in either oil. When the oils were tested using a microsomal enzyme preparation (S9), the results shown in Figure 1 were obtained. These results (Fig. 1) are in agreement with other studies (9-10,12) in showing that the crude Paraho shale oil is mutagenic and the hydrotreated shale oil is not detectably mutagenic towards Salmonella when tested in the presence

of rat liver microsomal enzymes (i.e., the Ames assay). Slope values (revertants/ug) for the responses shown in Figure 1 were calculated using data from initial linear regions of dose-response curves and are given in Table 1 (in the "Ames assay" column).

The photomutagenic responses of Salmonella suspensions to fluorescent light plus either the crude shale oil or the hydrotreated product oil are shown in Figure 2. The shale oils were tested at several concentrations and the mutation frequency responses are plotted in Figure 2 as a function of the product of shale oil concentration times duration of irradiation. The apparent dependency of the photomutagenic responses on the product of oil concentration times light exposure demonstrates a form of "reciprocity" of oil and light doses on the photomutagenic response, a phenomenon observed previously with an Eastern U.S. shale oil sample (34). The responses shown in Figure 2 were normalized to reflect the response to 100 ug/ml and slope values for linear fits of the mutation frequency responses as a function of minutes irradiated are given in Table 1. Based upon these slope values, hydrotreatment reduced photomutagenicity by approximately 78%.

Photomutagenicity of Hydrotreated Paraho Shale Oil Chemical Class Fractions

Column chromatography of the hydrotreated Paraho shale oil on neutral alumina (as described in refs. 36-38) yielded fractions termed A1, A2, A3 and A4 which were enriched in aliphatic hydrocarbons, neutral polycyclic aromatic hydrocarbons (PAH), nitrogen-containing polycyclic aromatic compounds (NPAC), and hydroxy-substituted PAC (HPAC) respectively. Fractions from several chemical class fractionations were bioassayed; the total recovery of material eluted by alumina column chromatography was > 84%. The percentage of the original material recovered in each fraction was: A1, 70-71%; A2, 10-11%; A3, 1%; and A4, 2-10%. (Values represent the range of recoveries from multiple determinations.) These results are in reasonably good agreement with values reported for a different separation procedure; i.e., 66.5% saturates, 9.8% PAH, 5.4% NPAC, 0.9% polars and 82.6% total recovery (45). The differences in recoveries of polar compounds may reflect an effect of alkyl substitutions (alkylation resulting in part from hydrotreatment) on chemical class separation schemes based upon the polarity of the sample. The A2-A4 fractions were each tested for photomutagenicity using 100 ug/ml; the mutation frequency responses are shown in Figure 3. Figure 3 shows that the A2 fraction was the most photomutagenic fraction, although all three fractions were active. The recovery of fraction A4 from the shale oil was highly variable (2-10%). The photomutagenicity of the A4 fraction was generally lower when the recovery of fraction A4 was low and the data for fraction A4 plotted in Figure 3 are from an A4 fraction in which high recovery was obtained. Slope values for mutation frequency responses as a function of minutes irradiated are given in Table 1.

Photomutagenicity of Coal Oils as a Function of Boiling Point Range

SRC-II distillation cuts having different boiling point ranges were tested for photomutagenic activity using 50 ug/ml of each oil (less the DMSO-insoluble component, see Materials and Methods). Slope values for mutation frequency responses as a function of minutes irradiated are given in Table 2. The data show a trend towards higher photomutagenic activity with increasing boiling point of the

material tested. In the absence of fluorescent light irradiation, the same SRC-II cuts were not detectably mutagenic, except the 800-850°F cut, which gave responses that were suggestive of a very low level of direct-acting (non-photosensitized) mutagenic activity. Treatment with 100 ug/ml of the 800-850° cut for two hours in the dark resulted in 34 revertants per plate above background.

Photomutagenicity of Coal Oil Chemical Class Fractions

The SRC-II 800-850° distillation cut was also separated into chemical class fractions by column chromatography on neutral alumina. Fractions from several separations were bioassayed; in these separations total recovery was ≥ 98%. The percentage of the original material recovered in each fraction was: A1, 3-4%; A2, 54-55%; A3, 23-25%; and A4, 17%; values that are in reasonable agreement with previously reported values (17,39,46). Fractions were prepared for bioassay as solutions in DMSO except fraction A1, which was insoluble in DMSO and was prepared as a DMSO "slurry" and as an aqueous solution with Tween 80. The slope values representing the mutation frequency response to fractions A1-A4 as a function of minutes irradiated are given in Table 2. The A2 fraction was clearly the most photomutagenic fraction; the A3 fraction was also photomutagenic, but the A1 fraction was inactive when tested either as a DMSO slurry or as an aqueous preparation with Tween 80. The A4 fraction was only slightly active or inactive. The SRC-II A1-A4 fractions were not detectably mutagenic when tested in the dark, except fraction A3, which induced 34 revertants per plate above background following two hours of exposure.

An SRC-I 800°+ distillate was also separated into fractions A1-A4; however, only the A2 fraction was soluble in the bioassay system. The A2 fraction was tested in a preliminary experiment (data not shown) and found to have no detectable mutagenicity in the dark; however, in the presence of light the A2 fraction was highly photomutagenic, showing a level of activity similar to that induced by the A2 fraction of the SRC-II 800-850° cut (Table 2).

DISCUSSION

Although a relatively limited number of samples have been tested, our data suggest that high boiling point components in the PAH-enriched fraction are the determinant chemical photomutagen(s) in synthetic fuels. Substantial photomutagenicity was also measured in coal oil and shale oil NPAC fractions and the HPAC shale oil fraction. The HPAC and aliphatic hydrocarbon fractions isolated from the SRC materials were relatively inactive. Strniste et. al. (31) has reported similar results in which essentially all the photomutagens (measured using cultured mammalian cells) present in a shale oil retort by-product water partitioned into a base- and neutral-enriched fraction.

Chemical analyses of PAH present in the materials tested are available in the literature (e.g., 17,18,45). Numerous PAH have been identified among the samples tested, including a variety of four- and five-ring compounds and alkyl derivatives thereof, and carcinogens such as benzo(a)anthracene, methylchrysenes, benzofluoranthenes and benzo(a)pyrene. It is not yet known which compounds present in the PAH fractions were responsible for photomutagenic activity. Benzo(a)pyrene has been reported to be photomutagenic in cultured mammalian cells (32) and UV radiation can enhance (and

inhibit) the carcinogenic response of mammalian cells to benzo(a)-pyrene (47). The occurrence of carcinogens and mutagens such as benzo(a)pyrene in samples that were not mutagenic with enzyme activation (SRC-II 800-850° A2 fraction, SRC-I 800°+ A2 fraction, hydrotreated Paraho shale oil, see Tables 1 and 2) suggests the presence of antimutagens in these samples. Haugen and Peak (48) have shown that undefined components can inhibit the microsomal enzyme activation of mutagens in coal liquids. Some heteroatomic compounds can also be expected to be present in the A2 fractions. For example, an SRC-II heavy distillate (from which the 800-850°F cut was in part derived) has been shown to contain three and four ring thiophene analogs, as well as dibenzofuran and methyldibenzofuran (30). Nitrogen heterocyclics identified in the hydrotreated Paraho shale oil include alkylated and unsubstituted carbazoles, benzocarbazole(s), and azapyrene(s) (36). The NPAC fraction of the SRC-II 800-850°F cut has been reported to contain a high concentration of benzo(a)carbazole (20.5 mg/g) and lower amounts of azapyrenes, 2-azafluoranthene, and numerous amino-substituted PAH (17).

This study was conducted in part to determine the relationship between mutagenic and carcinogenic activities in complex mixtures such as synthetic fuels. Tables 1 and 2 give data indicative of carcinogenicity, photomutagenicity, and mutagenicity in the presence of microsomal enzymes (Ames assay) for shale oil (Table 1) and coal oil (Table 2) materials. Both the crude and hydrotreated Paraho shale oil samples have been reported to be carcinogenic following chronic dermal applications to mouse skin. Hydrotreatment was reported to reduce but not eliminate carcinogenicity (7-10), and the carcinogenic potencies of the crude and hydrotreated Paraho shale oils given in Table 1 are relative to the response to benzo(a)pyrene (8). The carcinogenic potencies of the coal oil materials given in Table 2 were derived from chronic and/or initiation-promotion (IP) mouse skin painting tests. The carcinogenicity results and the mutagenicity with enzyme activation (i.e., Ames assay results using strain TA98) for the coal oils (Table 2) have been normalized to give responses relative to the response to an SRC-II heavy distillate, arbitrarily given a value of 100 (39).

A comparison of the carcinogenic and mutagenic activities given in Tables 1 and 2 should be approached with caution. The data in Tables 1 and 2 were to some degree derived by procedures that provide estimates and different analyses were apparently used to arrive at the carcinogenicity values given for the shale oils, the SRC-II distillates and the SRC-II 800-850° chemical class fractions. A cautious approach for comparing the bioassay results would be to consider only whether a test substance produced positive or negative results. The most obvious discrepancy between assays was observed with the SRC-II 800-850° A2 fraction (Table 2), which was highly carcinogenic and highly photomutagenic but was not mutagenic with enzyme activation. Similarly, an SRC-I 800°+ A2 fraction (not shown in Table 2) was observed to be highly carcinogenic in the initiation-promotion test (49), highly photomutagenic in a preliminary test (see Results), but not mutagenic with enzyme activation (19). The hydrotreated Paraho shale oil (Table 1) was also found to be positive for carcinogenicity and photomutagenicity but not mutagenic with enzyme activation (9,10,12, Table 1), although one study (50) reported a low level of enzyme-mediated mutagenicity (0.24 revertants/ug) for this sample. The SRC-II 300-700° cut (Table 2) elicited an apparently "false positive" response, being apparently

negative for carcinogenicity and photomutagenicity but positive for enzyme-mediated mutagenicity. Another report (51) also found this sample to be mutagenic with enzyme activation, although two others (18,46) did not. Of the remaining samples, the crude Paraho shale oil, the SRC-II 700-750°, 750-800°, and 800-850° cuts and the 800-850° A3 fraction were all positive for carcinogenicity, photomutagenicity, and mutagenicity with enzyme activation; the SRC-II 800-850° A1 and A4 fractions were all negative in the three bioassays or gave responses that were suggestive of a low level of activity.

In summary, of 11 samples that have been tested for carcinogenicity, photomutagenicity and enzyme-mediated mutagenicity, in 7 cases there was agreement between all three assays and in 4 cases the photomutagenicity data was in better qualitative agreement with carcinogenicity than were the mutagenicity data obtained using enzyme activation. The strong agreement between photomutagenicity and carcinogenicity among the synfuel materials could be coincidental. However, the agreement is sufficiently extensive to consider possible fundamental underlying relationships. It is possible that animals exposed to the synfuels were also exposed to significant amounts of environmental radiation (such as fluorescent room light), and the positive correlation shown in Table 2 may reflect a mediation of coal oil-induced tumorigenesis by photochemical processes. Alternatively, photosensitized effects caused by chemicals such as those present in synthetic fuels may have served as a selective pressure for the evolution of the enzymes that degrade photosensitizers and apparently have a role in PAC-induced carcinogenesis. It is also possible that photomutagenesis and carcinogenesis by PAC synfuel components proceed by the same mechanism(s). One possible common mechanism could involve the participation of reactive oxygen species such as superoxide anion, which is generated 1) by endogenous cellular chromophores when irradiated with near UV light (52), 2) in human lungs in response to chronic tobacco smoke exposure (53), and 3) possibly also in mouse skin following treatment with synfuel materials.

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Table 1. Carcinogenicity and Mutagenicity of Shale Oils

Material		Carcinogenicity[a] (chronic test)	Mutagenicity Ames[b]	Mutagenicity Photomutation[c]
Crude Paraho Shale Oil		1/998	3.7±0.1	45±20
Hydrotreated Paraho Shale Oil		1/2780	-0.0004±0.0009	10±1.7
Hydrotreated Paraho Shale Oil Chemical Classes	A2	ND[d]	ND	48±9.0
	A3	ND	ND	39±8.2
	A4	ND	ND	32±7.8

[a]From ref. 8, carcinogenic potencies relative to benzo(a)pyrene.
[b]This study, revertants/ug oil.
[c]This study, revertants/10^9 survivors/minute irradiated, 100 ug/ml tested or normalized to give responses to 100 ug/ml.
[d]ND-not determined.

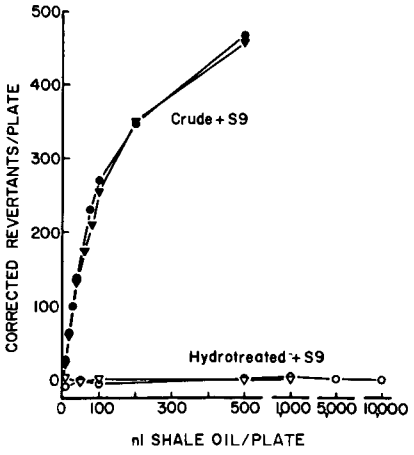

Figure 1. Mutagenicity of crude (closed symbols) and hydrotreated (open symbols) Paraho shale oils tested in two separate experiments (circles and triangles) with a microsomal enzyme preparation (S9).

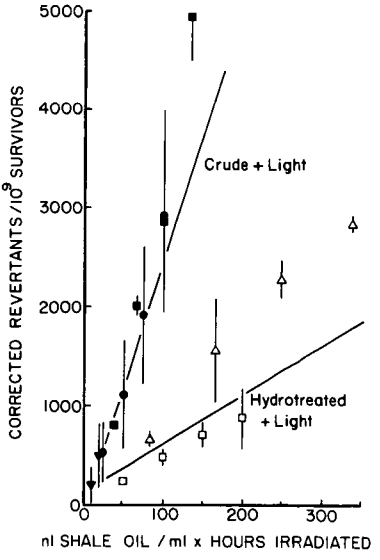

Figure 2. Photomutagenicity of crude (closed symbols) and hydrotreated (open symbols) Paraho shale oils tested using 10 (▽), 50 (○), 100 (□), or 250 (△) nl oil/ml.

Table 2. Carcinogenicity and Mutagenicity of SRC-II Distillates and Chemical Classes

Material	Relative Carcinogenicity IP[a]	Relative Carcinogenicity Chronic[a]	Relative Mutagenicity Ames[a]	Relative Mutagenicity Photomutation[b]
Distillates:				
Heavy distillate	100	100	100	ND
300-700°F	0	0	70	2.0 ± 2.3
700-750°F	18	87	95	12 ± 9.3
750-800°F	14	120	138	15 ± 3.7
800-850°F	49	157	148	27 ± 2.2
Chemical classes:				
800-850°fraction A1	10[c]		0	1.3 ± 3.8
A2	158		0	200 ± 101
A3	79		700	32 ± 9.2
A4	---[c]		0	6.6 ± 7.7

[a]From ref. 39. Values are relative to heavy distillate, given a value of 100. IP - initiation-promotion test.
[b]This study, revertants/10^9 survivors/minute irradiated, 50 ug/ml tested. ND - not determined.
[c]Not a significant response.

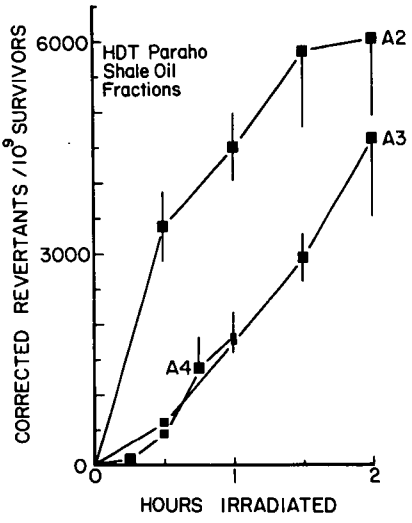

Figure 3. Photomutagenicity of hydrotreated Paraho shale oil chemical class fractions tested at 100 ug/ml.